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Final Technical Report

Subject: Research on the production and immunological examination of artificial antigens containing known sugars (or oligo-saccharides) as the determinant groups, in relation to the immunochemical analysis of enterobacterial O-antigens (endotoxins).

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Chief investigator: Professor Otto Westphal, D.Sc.

Basis and Summary

In the Final Report of November 30, 1961, the principal research lines were characterized. Enterobacterial O-antigens are complex toxic lipopolysaccharides on the surface of the bacterial cell wall. Their specificity is given by oligosaccharidic side chains of the highly branched polysaccharide components. By serologic inhibition techniques the determinant endgroups of various O-antigen factors were identified. It was found that 3, 6-dideoxy-hexoses frequently play a role as endgroups of important determinants in O-antigens of highly pathogenic strains, such as Salmonella paratyphi, Salmonella typhi, Escherichia coli 111, and many others.

It could be demonstrated (O. LÜDERITZ, O. WESTPHAL, A. M. STAUB and L. LE MINOR, Nature (London) 186, 556-558(1960)) that in certain instances single monosaccharides, in the form of determinants of artificial antigens, already represented a large part of the determinant group of the related natural bacterial antigen. It was shown that the artificial antigens were non-toxic, in contrast to the bacterial O-antigens, which are part of the so-called endotoxic complex.
Consequently, the following programme was developed in view of theoretical as well as practical aspects:

1) Further elaboration of determinant oligosaccharide structures of enterobacterial O-antigens.

2) Synthesis of determinant sugars or oligosaccharides; improvement of already existing procedures.

3) Use of these sugars or suitable derivatives (such as glycosides of known stereochemical configuration) for serologic inhibition techniques in systems of bacterial O-antigens and precipitating antisera, preferably absorbed monospecific antisera.

4) Production of non-toxic artificial antigens with serologically active sugars and immunization of animals. Investigations on the nature of antibodies provoked by immunization with these artificial antigens, especially with respect to their possible antibacterial or anti-infective activities.

As a general basis of these investigations see O. WESTPHAL and O. LÜDERITZ, Chemic bakterieller O-Antigene (Chemistry of Bacterial O-Antigens), Pathologia et Microbiologia 24, 870-889(1961).

During the period of December 1961 to November 1962 the following investigations were performed:

(A) The structure of determinant groups of O-antigens of pathogenic Enterobacteriaceae, especially of Salmonella and certain Escherichia strains was further eleborated, using .
1. Serologic inhibition techniques (collaboration with Dr. A. M. STAUB, Pasteur Institute, Paris),
2. Chemical and immunochemical techniques.

(B) p-Nitrophenyl-glycosides of determinant sugars were prepared and reduced to the corresponding p-aminophenyl-glycosides, which were coupled to suitable proteins to give the sugar-phenylazo-proteins as artificial antigens with the very sugar as determinant endgroup. The influence not only of the determinant sugar on specificity, but also of the carrier protein and its degree of substitution on antigenicity of the sugar-phenylazo-protein(s) was studied (partly in collaboration with Dr. M. SELA, Weizmann Institute, Rehovoth/Israel).

(C) Artificial antigens, especially with 3. 6-dideoxy-hexoses - colitose (3-deoxy-L-fucose), abequose (3-deoxy-D-fucose) and tyvelose (3-deoxy-D-rhamnose) - were injected into various animal species - mice, rabbits, goats - and the response of the immunized animals was followed qualitatively and quantitatively.

(D) In view of the fact that single terminal sugars of determinant groups of bacterial polysaccharide antigens certainly do not represent the whole specific structure, research was also started to couple non-toxic degraded O-specific bacterial polysaccharides with proteins. (The ideal along these lines would probably be the synthesis of determinant di- or trisaccharides and their coupling to proteins. Research on the synthesis of certain determinant disaccharides is being started).

(E) For a better understanding of the reactions and cross-reactions of deoxyhexoses with antibodies, a series of closely related hexoses and their nitrophenyl-α-glycosides were synthesized. The series is based on D-galactose, of which the 2-, 3- and 6-deoxy-derivatives were prepared, whilst 4-deoxy-D-galactose is under investigation. 2, 6-dideoxy- and 3, 6-dideoxy-D-galactose (the latter one being abequose) were also synthesized.

(F) A new group of biologically active mucopolysaccharides were isolated from various E. coli strains by Dr. K. and Mrs. B. Jann (Max-Planck-Institute, Freiburg). These mucopolysaccharides show very high pyrogenicity, comparable to the endotoxic lipopolysaccharide of the same strain, but proved to be much less toxic. Pyrogenicity and toxicity can thus be differentiated. Structures, common to all bacterial pyrogenes appear to be composed of D-glucosamine, ß-hydroxy-myristic acid and labile O-acetyl groups. Related sub-units, such as ß-hydroxy-myristinoyl-D-glucosamine and further derivatives, were synthesized.

Mass cultivation of many bacterial species (serotypes) were again done by Professor F. KAUFFMANN and also by Drs. F. and I. ØRSKOV of Statens Serum-institut, Copenhagen, and by Dr. H. HURNI, Microbiological Research Dept. of the WANDER S. A., Berne. Certain bacterial extracts were placed at our disposition by Dr. A. M. STAUB of the Pasteur Institute, Paris, and a 3kg-batch of Serratia marcescens (from mass cultures) was given to us by courtesy of Dr. F. W. HOFFMANN of Army Chemical Center, Maryland.

I. The Glycosidic Linkage of Terminally Bound 3, 6-Dideoxy-hexoses in Bacterial Polysaccharides

As indicated in the last Final Report of Nov. 1961, the isolation of terminal oligosaccharides containing 3, 6-dideoxy-hexoses is not possible because of the acid-lability of the glycosidic linkage of the latter ones. By serologic inhibition techniques, using defined α- and ß-glycosides it is however possible to estimate the nature of the linkage in the natural polysaccharide antigen. In the last Final Report the following glycosides were listed and their synthesis described:

Methyl-α-abequoside-(1, 5)
p-Nitrophenyl-α-abequoside-(1, 5)
" -ß- " -(1, 5)
Methyl-α-tyveloside-(1, 5)

p-Nitrophenyl-α-colitoside-(1, 5)
" -ß- " -(1, 5),

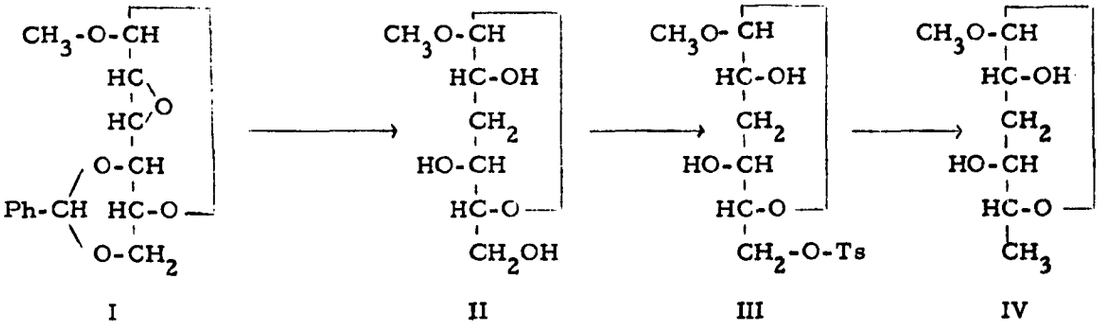
and typical inhibition experiments were given. The synthesis of the following further abequoside and tyvelosides could be achieved:

Methyl-ß-abequoside-(1, 5)

Methyl-ß-tyveloside-(1, 5)

p-Nitrophenyl-α-tyveloside-(1, 5)

Methyl-ß-abequoside-(1, 5). - 2, 3-Anhydro-4, 6-benzylidene-ß-methyl-D-gulopyranoside (I) was hydrogenated with Raney nickel at 100°C/100 atm. H_2, following the procedure of H. HUBER and T. REICHSTEIN, Helv. Chim. Acta 31, 1645(1948), to give ß-methyl-3-deoxy-D-xylo-hexopyranoside (II; = ß-methyl-3-deoxy-D-galactoside). II could be clearly characterized as 3-deoxy-galactose derivative. II was partially tosylated, and the pure 6-tosyl derivative (III) was separated by chromatography. Reduction of III was performed directly (and not over the 6-iodo derivative) using $LiAlH_4$, giving ß-methyl-3, 6-dideoxy-D-xylo-hexopyranoside (IV; = ß-methyl-abequoside-(1, 5)) in good yield, which was highly purified by chromatography.



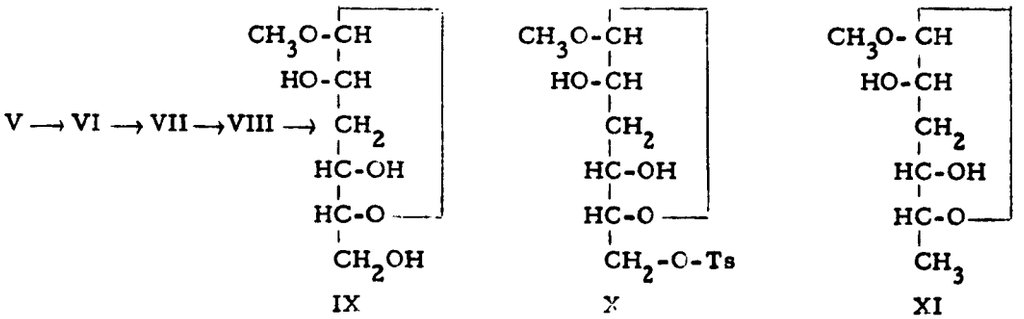
I II III IV

Serologic Inhibition. - Abequose, α- and ß-methyl-abequoside-(1, 5) were tested as inhibitors of the precipitation of S. paratyphi B antiserum (factors anti-4, -5 and -12) with S-paratyphi B polysaccharide. For a more specific reaction, the bacterial polysaccharide was pretreated with periodate leaving terminal abequose units (factor 4) intact (because of the lack of 1, 2-glycolic hydroxyl groups), but destroying practically all other determinant factors (terminal glucose, 2-O-acetyl galactose, and rhamnose). See A. M. STAUB, R. TINELLI, O. LÜDERITZ and O. WESTPHAL, Ann. Inst. Pasteur 96, 303-332 (1959). The results are listed in Table I. Comp. last Final Rep., pg. 6, Table 3.

Inhibitor	Inhibition (%) with 2	10	50µMol
Abequose	18	27	32 (1961)
Methyl-α-abequoside-(1, 5)	13	35	43 (1962)
Methyl-ß-abequoside-(1, 5)	11	22	30 (1962)

Table 1: Relative Inhibiting Capacity of Abequose, Methyl-α- and -ß-abequoside-(1, 5) in the Precipitating System of S. paratyphi B Antiserum with S. paratyphi B Polysaccharide (Periodate-oxidized)

Methyl-ß-tyveloside-(1, 5). - The synthesis of this compound could be completed over ß-methyl-3-deoxy-D-mannopyranoside (IX). ß-Methyl-4, 6-benzylidene-D-mannopyranoside (V) was tosylated with 1 mol tosylchloride/pyridine to give a mixture of the 2- and 3-tosyl derivatives, which could be separated and crystallized. By treatment of ß-methyl-2-tosyl-4, 6-benzylidene-D-mannoside (VI) with sodium methylate in methanol the 2, 3-anhydro derivative (VII) was obtained in crystalline from (m. p. 186-187°, $[\alpha]_{5461}$ = -28.5°). Mild reduction with $LiAlH_4$ gave ß-methyl-3-deoxy-4, 6-benzylidene-mannoside (VIII) which was converted into ß-methyl-3-deoxy-D-mannopyranoside (IX) by hydrolysis with 80 p. c. acetic acid at 80°C. IX was tosylated to give the 6-tosylate (X) which could be purified by chromatography. Finally X was again treated with $LiAlH_4$ to give ß-methyl-3, 6-dideoxy-D-arabino-hexopyranoside (XI; = methyl-ß-tyveloside); $[\alpha]_{5461}$ = -72°.



Serologic Inhibition. - Recently tyvelose, α- and ß-methyl-tyveloside-(1, 5) were tested (partly again; comp. 3rd Quart. Report of August 31, 1962) as inhibitors in the precipitating system of S. typhi horse antiserum with S. typhi polysaccharide (pretreated with periodate; see above). The results are shown in Table 2.

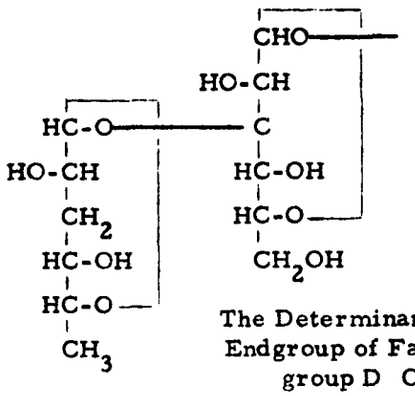
Inhibitor	Inhibition (%) with		
	0, 4	2	10 µMol
Tyvelose	10	22	45
Methyl-α-tyveloside-(1, 5)	21	37	71
Methyl-ß-tyveloside-(1, 5)	7	15	34

Table 2: Relative Inhibiting Capacity of Tyvelose, Methyl-α- and ß-tyveloside-(1, 5) in the Precipitating System of S. typhi Antiserum and S. typhi polysaccharide (periodate-oxidized)

p-Nitrophenyl-α- and p-Aminophenyl-α-tyveloside-(1, 5). - As shown in the 2nd and 3rd Quarterly Report p-nitrophenyl-α- and p-aminophenyl-α-tyveloside-(1, 5) were prepared by aid of the usual procedure: tyvelose ⟶ triacetyl-tyvelose ⟶ p-nitrophenyl-diacetyl-α-tyveloside-(1, 5) ⟶ p-nitrophenyl-α-tyveloside-(1, 5) ⟶ p-aminophenyl-α-tyveloside-(1, 5).

In serologic inhibition tests with tyvelose, methyl-α-tyveloside-(1, 5) and p-aminophenyl-α-tyveloside-(1, 5), using an analogous precipitating system as indicated in Table 2, the p-aminophenyl-α-tyveloside-(1, 5) - as could be expected - proved to be an even better inhibitor as compared to tyvelose or methyl-α-tyveloside.

From these experiments (see also Table 2) it can be clearly concluded that tyvelose in the S. typhi O-antigen is bound terminally and in α-pyranosidic linkage. (The ß- and furanosidic linkages could be excluded). - There ist much indication (collaboration with Dr. A. M. STAUB, Paris) that tyvelose in S. typhi and other Salmonella group D polysaccharides is bound to the 3-position of D-mannose. Therefore, the determinant disaccharide endgroup of Factor 9 (KAUFFMANN-WHITE scheme) of Salmonella group D O-antigens has the structure of tyvelopyranosido-α-1, 3-D-mannopyranoside---(XII):



The Determinant Disaccharide Endgroup of Factor 9 in Salmonella group D O-Antigens

XII

Most of the synthetic work in the field of 3, 6-dideoxy-hexoses and derivatives was summarized in the Thesis of Dr. S. STIRM (Freiburg, 1962) "Beiträge zur chemischen Strukturanalyse einiger O-Antigene der Salmonellen" (94 pg.), 5 copies of which were delivered to the European Research Center.

Linkage of 3, 6-Dideoxy-hexoses in Enterobacterial O-Antigens. - It is known that 3, 6-dideoxy-hexoses are acting as determinant endgroups of O-antigens of many pathogenic enterobacterial strains. See for instance O. WESTPHAL and O. LÜDERITZ, Pathologia et Microbiologia 24, 870-889 (1961). On the basis of inhibition studies with 3, 6-dideoxy-hexoses and glycosides of known structure it was found that in all hitherto investigated polysaccharides 3, 6-dideoxy-hexoses, acting as determinant endgroups, are bound in α-pyranosidic linkage. This is true for

Abequose in factor 4 of Salmonella group B,
Tyvelose " " 9 " " " D,
Colitose " " 35 " " " O, and
Colitose in Escherichia coli O 111:B4.

The second hexose unit in the determinant oligosaccharides of O-antigens, to which the respective 3, 6-dideoxy-hexose is attached (besides factor 9 of Salmonella group D), is

Colitose-α-1, 4-D-glucose--- in factor O 35 of Salmonella group O.

The same structure, with an additional D-galactose in the 3rd position, is true for the determinant group of E. coli O 111, namely

Colitose-α-1, 4-D-glucose-α-1, 4-D-galactose---.

The linkage of the 3, 6-dideoxy-hexoses in further enterobacterial O-antigens is still under investigation. In the structural research work the main chemical principle followed is the analysis of fully methylated O-antigenic polysaccharides before and after splitting off 3, 6-dideoxy-hexose (which is bound acid-labile). - The main immunochemical principle is the serological analysis of the partially degraded polysaccha-

ride for the new specificity (set free by splitting off the terminal 3. 6-dideoxy-hexose). (Publication of results with E. coli O 111 is in preparation).

Mild acid hydrolysis of the E. coli O 111 polysaccharide leads to a partially degraded polysaccharide of nearly the original molecular weight (ultracentrifuge) but with a very low content of colitose. The degraded polysaccharide reacted with Pneumococcus type V antisera (done by Professor M. HEIDELBERGER, New Brunswick, N. J. /USA) and with absorbed Salmonella O 12_2-antiserum, proving that by splitting off terminal colitose endgroups new endgroups of D-glucosido-α-1. 4-D-galactose-- were set free. - The synthesis of the disaccharide D-glucosyl-α-1. 4-D-galactose, which has not been prepared hitherto, is now under investigation.

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II. Immunization with Artificial Antigens Containing 3. 6-Dideoxy-hexoses as the Determinant Endgroup

Until now artificial antigens with α- and ß-colitose (see Nature 186, 556-558 (1960), α-abequose and recently with α-tyvelose (see 3rd Quarterly Report of Aug. 31, 1962) have been prepared by aid of the p-aminophenyl-3. 6-dideoxy-hexosides and coupling to suitable proteins via Landsteiner's azo method. The following immunizations were tried:

Artificial Antigen with	Injected Animals
α-Colitoside Endgroups	Rabbits Goats Mice
α-Abequoside "	Rabbits Goats Mice
α-Tyveloside "	(Rabbits) Goats

Results with the α-Abequose antigen were as follows:

1. Rabbits and goats reacted with the production of anti-abequose antibodies, which however did not cross-react with abequose-containing bacterial polysaccharides (precipitation, passive hemagglutination).
2. Experiments, using 1000 mice (Dr. H. HURNI, Berne) showed that immunization with abequose antigens, with or without adjuvant, did not stimulate a significantly increased protection against S. typhimurium infection. A non-significant slight increase for the abequose-immunized animals in comparison to the controls was stated. But S. typhimurium vaccine was significantly better. Therefore, no detailed serological analyses of individual and/or pooled sera were done.

Results with the α-Tyvelose antigen (Tyvelose-α-phenylazo-BSA) were as follows:

All of 5 immunized goats reacted with the production of anti-tyvelose antibodies. All antisera precipitated S. typhi polysaccharide. Sera taken before immunization did not show any antibodies of anti-tyvelose specificity, nor did they show anti-Salmonella group D antibodies. In contrast to the rather high precipitin titers, passive hemagglutination tests indicated only low anti-S. typhi hemagglutinins (which was unexpected and is hitherto unexplained).

In conclusion: From those animals hitherto immunized with artificial 3, 6-dideoxy-hexose antigens, only goats reacted with the production of anti-colitose and anti-tyvelose antibodies, which also showed certain antibacterial activity. α-Abequose antigens, until now, neither in goats nor in rabbits or mice, gave rise to any antibacterial antibodies.

From these experiments it can further be concluded that artificial antigens with 3, 6-dideoxy-hexoses as monosaccharidic determinants only occasionally and only in certain species (goats) elicite anti-3, 6-dideoxy-hexose antibodies which at the same time also show specific reactivity towards related bacterial O-antigens. Generally the monosaccharidic determinants do not suffice to be comparable with the potency of the bacterial O-antigenic determinants in vaccines. Therefore, oligosaccharidic structures, especially determinant disaccharides, have to be taken into consideration. The preparation of such oligosaccharides appears to be of considerable interest. The synthesis of disaccharides and their p-aminophenyl-glycosides is now being tried.

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III. Coupling of Degraded Bacterial Polysaccharides to Proteins (Artificial O-Antigens)

As pointed out in Section II, monosaccharidic determinants generally do not suffice to give artificial antigens provoking the production of effective antibacterial antibodies. As long as specific disaccharides are not available, the natural bacterial polysaccharides are the only source of determinant oligosaccharidic structures. In an attempt to find out as to whether determinant oligosaccharides are immunologically moreeffective as compared to single determinant sugars (endgroups), various non-antigenic and non-toxic degraded enterobacterial polysaccharides were prepared, converted into coupling derivatives and coupled to proteins to give the polysaccharide-O-benzyl-azo-proteins.

As outlined in the 3rd Quart. Report (Aug. 31, 1962) the degraded (lipid-free and non-toxic) polysaccharides were extracted from suitable enterobacterial organisms using the method of G. G. FREEMAN, Biochem. J. 36, 340(1942). It was found that they could be further purified by phenol followed by hydrazine treatment. Hydrazine could be separated by extraction with benzaldehyde. In this manner highly purified degraded polysaccharides were obtained, free of amino acids and other non-polysaccharide constituents (mol. weight about 20-30,000). These polysaccharides nevertheless reacted strongly with homologous O-antisera showing that the determinants were still intact. Only acid- or alkali-labile groups are destroyed, such as the O-antigen factor 5 of Salmonella group B, the terminal sugar of which is 2-O-acetyl-D-galactose after A. M. STAUB .

It was found that the introduction of p-nitrobenzyl groups can best be accomplished by treatment of the polysaccharide, dissolved in dimethylsulfoxide (DMSO), with nitrobenzyl bromide and silver oxide. Every degree of substitution of the polysaccharide with nitrobenzyl ether groups could be realized in a reproducible form. - The whole procedure was first exercised using degraded starch as a model polysaccharide. For details see U. HÄMMERLING, "Synthese künstlicher Antigene mit bakterieller O-Spezifität", chemical diploma thesis, Freiburg (1962). One copy is attached to the present Technical Report.

Precipitation of the unsubstituted and the substituted polysaccharides showed that even high degrees of substitution did not significantly impair with the specific reactivity towards antisera. The following polysaccharides were converted into p-nitrobenzyl ethers of various degrees of substitution:

Salmonella adelaide polysaccharide
Salmonella typhi "
Escherichia coli O 111 "

After reduction to the p-aminobenzyl ethers the polysaccharides were diazotized in the usual manner and coupled to proteins.

Special attention was given to the protein carrier. Recently Dr. G. NOSSAL of the W. and E. Hall Institute for Med. Research (Director Sir Macfarlane Burnet) in Melbourne made the observation that highly purified flagella protein of Salmonella strains, injected into rats and rabbits, gave rise to extremely high titers of anti-O-antibodies, although the flagella protein (H antigen) containes less than 1-2 p. c. of polysaccharide (O-antigen). As a possible explanation of this effect it can be assumed that the special long shape of the flagella protein molecule acts as a very effective antigenic carrier for the O-specific polysaccharide hapten. Therefore, proteins of long thread-like shape were also included in our studies.

These proteins are for instance synthetic polypeptides, developed by Dr. M. SELA of the Weizmann Institute in Rehovoth/Israel). See M. SELA's excellent article: "Studies on the Chemical Basis of the Antigenicity of Proteins", Biochem. J. 85, 223-235(1962). Collaboration with Dr. Sela and his group was started in 1962, and various synthetic multi-chain poly-aminoacids were placed at our disposition and used as carriers for model monosaccharides (D-galactose) and some of the above mentioned polysaccharides. A preliminary principal finding is that antigens with the synthetic proteins show much less carrier-specific antibodies in contrast to antigens with natural protein carriers, such as ovalbumin, serum albumin etc., although the proportion in both types of antigens was practically the same. Work is being continued.

An artificial antigen, produced by coupling the p-aminobenzyl ether of the degraded Salmonella typhimurium polysaccharide to bovine serum albumin (BSA), is now tested for the immunogenic response of mice (comp. Section II: abequose antigen). Further antigens are being prepared and tested in collaboration with Dr. M. SELA.

The main question to be answered is: Is the toxic component of the endotoxic complex of Enterobacteriaceae a necessary adjuvant for the O-antigen, which is firmly bound to the complex? Or can the polysaccharide and protein - without the toxic structures of the complex - be recombined to obtain a non-toxic and effective antigen? Further work is devoted to a possible answer of these questions.

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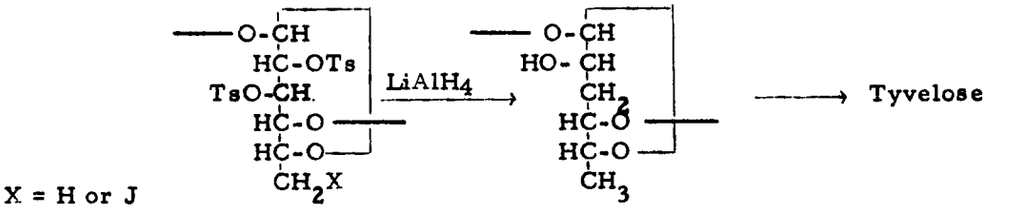
IV. Preparation of Poly-3, 6-dideoxy-hexoses etc.

This subject was extensively discussed in the 3 foregoing Quart. Reports of 1962 (see 1st Rep., pg. 4 - 2nd Rep., pg. 4 - 3 rd Rep., pg. 4). Work was done in collaboration with Dr. W. J. WHELAN, London.

The present state of these investigations can be summarized as follows:

1. Amylose can be converted into 6-deoxy-amylose (poly-6-deoxy-glucose). 6-Deoxy-amylose can further be converted into a poly-3, 6-dideoxy-hexose. Originally we assumed that the 3, 6-dideoxy-hexose is paratose, and the polysaccharide would thus be poly-paratose with ß-1, 4-linkages. In attempts to isolate the 3, 6-dideoxy-hexose from hydrolyzates of the polysaccharide it was found that its optical rotation, $[\alpha]_D^{20} = +25^\circ$, did not correspond with the figure for bacterial paratose, but was exactly the rotation found for bacterial tyvelose. A sample of the 3, 6-dideoxy-hexose was tested for serological inhibition. It showed a strong inhibition of a Salmonella O 9/ anti Salmonella O 9 system (S. typhi/antiserum), clearly indicating that the 3, 6-dideoxy-hexose was indeed tyvelose. The reduction of 2, 3-ditosyl-6-iodo- or 2, 3-ditosyl-6-deoxy-amylose (XIII) with $LiAlH_4$ in tetrahydrofurane gave rise to an inversion at C-atom 2, so that the D-glucose configuration is converted into the D-mannose configuration (XIV):



X = H or J

XIII XIV

Starting from amylose the synthesis therefore leads to a poly-tyvelose (XIV). A larger batch of this poly-tyvelose has been prepared for further immunological studies (antigenic response, specificity of antibodies ?).

Attempts to improve the method in a manner that pure tyvelose could be obtained in larger amounts and in a comparatively economic manner so far failed. The yield of pure tyvelose does not exceed about 10-15 p. c. of the weight of the starting amylose, and the whole process is rather time-consuming.

2. Mannan and galactan were subject of the same procedure. Because of their insolubility in the usual solvents, these polysaccharides were first formylated and the formyl-polysaccharides then tosylated. After splitting off the formyl ester groups, the partially tosylated polysaccharides were fully tosylated to give nearly analytically pure

tri-tosyl-polysaccharides. However, the treatment with $LiAlH_4$ did not lead to 3. 6-dideoxy-hexoses. Until now, no 3. 6-dideoxy-hexoses could be isolated from the hydrolyzates of these polysaccharides. Work is being continued, also making use of monosaccharidic model substances, such as 2. 3-ditosyl-6-deoxy-D-mannosides and -D-galactosides and other suitable derivatives. It is our intention to finish these studies in due time, after the results obtained so far do not appear to be too promising.

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IV. A. Polycondensation of Activated Monosaccharides for the Production of Synthetic Polysaccharides

As mentioned in the 2nd Quart. Report (of May 31, 1962) a new condensation procedure for hexose-1-polyphosphates was developed by G. SCHRAMM et al. (Angew. Chemie 74, 53-59(1962)). Professor G. Schramm asked us for collaboration in the field of polysaccharide synthesis (because he wanted to concentrate on the synthesis of nucleic acids by aid of the same principal procedure; see G. Schramm, l. c.).

In preliminary experiments, we were able to reproduce the preparation of poly-D-glucose, according to Schramm's method, with a yield of about 15 p. c. on the basis of the starting glucose. x) The mol. weight of the obtained glucans is now being estimated. It was also found that other hexoses (mannose, galactose) and even pentoses (xylose, ribose) could be subjected to poly-condensation. At present the production of mixed polymers is being tried. In principle, the method of Schramm is working. Our main interest would be in two directions: (a) poly-condensation of deoxy- and dideoxy-hexoses, and (b) experiments on the possibility of linking certain activated sugars to the ends of chains of preformed oligo and/or polysaccharides. After dimethylsulfoxide (DMSO) was found to be an excellent solvent, even for various branched polysaccharides (see Section III), it appears possible to elaborate procedures of this sort. Work is now under way, and the chemical and immunological nature of sugar-polymers is being investigated.

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x) In the meantime G. SCHRAMM et al. found (as we did also) that the D-glucose polymer does not only contain ß-1. 4-linkages, but certainly also ß-1. 6- and possibly other glycosidic linkages.

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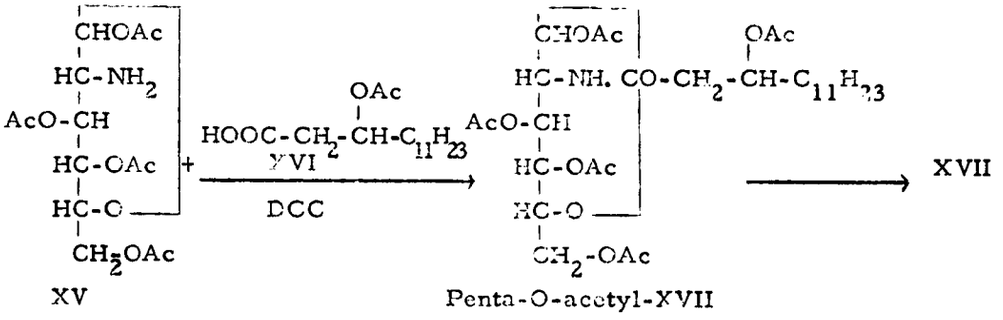
V. The Synthesis of Long-Chain Hydroxy-Fatty Acid Derivatives of D-Glucosamine

Enterobacterial O-antigens are part of the endotoxic complex (see for instance O. WESTPHAL, O. LÜDERITZ and A. M. STAUB, Bacterial Endotoxins, J. Med. and Pharm. Chem. 4, 497-504(1961); O. WESTPHAL, Ann. Inst. Pasteur 98, 789-813(1960)). A common component of many (if not all) endotoxins is a certain lipid, called lipid A. Lipid A is a derivative of D-glucosamine, long-chain fatty acids, O-acetyl and phosphoric acid ester. It is rather firmly bound to the O-specific polysaccharide (as a lipopolysaccharide).

Very recently we discovered in many Escherichia strains, analyzed for O- and the so-called K-antigens, a new type of acid mucopolysaccharides (work of Dr. and Mrs. K. JANN, granted by the Deutsche Forschungsgemeinschaft and the Fraunhofer-Gesellschaft). These acid mucopolysaccharides (MPs) proved to be as pyrogenic as the O-antigenic lipopolysaccharide (LPs) of the same strain. In toxicity tests (mice) it was however found that the MPs were much less toxic than the LPs. This indicates that pyrogenicity and toxicity can be differentiated. In the acid mucopolysaccharides no typical lipid A component could be detected. But there are still certain typical constituents of lipid A present, namely:

D-glucosamine,
ß-hydroxy-myristic acid, and
labile O-acetyl.

The ß-hydroxy-myristic acid was found to be bound amide-like to the amino group of D-glucosamine. This is also true for typical lipid A of endotoxic lipopolysaccharides. Therefore N-ß-hydroxy-myristinoyl-D-glucosamine with additional O-acetyl groups appears to be a common structure of all hitherto isolated bacterial pyrogens and may be responsible for (or involved in) pyrogenicity. For this reason ß-hydroxy-myristinoyl-D-glucosamine (XV) was synthesized. Also acetylated derivatives were prepared. For the synthesis condensation of tetra-O-acetyl-D-glucosamine (XV) with ß-acetoxy-myristic acid (XVI) was condensed with di-cyclohexyl-carbodiimide (DCC) as condensing agent.



Such and further derivatives of D-glucosamine will be tested for possible pyrogenic and other "endotoxic" activities (or blocking of such activities). Synthetic work is being continued.

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Scientific personal taking part in the investigations of this Contract:

Karl HIMMELSPACH, D. Sc.

Stefan STIRM, D. Sc.

1 Technical Assistant

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Coworkers (excluding the Chief Investigator) devoted nearly 7000 manhours to this contract. In carrying out the contract we expended exactly DM 11. 600. -- for materials and overhead items. No important property was acquired during the contract period. The technical equipment of the Institute and the library were at the disposition of the group without any costs besides the calculated depreciation charge of DM 1000. - for the equipment used.

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Statement of the finances used for the whole period of this contract:

Amount of Contract Found ..... (Including Modification Nr. One (1))		DM 55. 300. -
Salaries	DM 38. 500. -	
Administrative Services	DM 4. 200. -	
Materials	DM 6. 200. -	
Overhead Charges	DM 5. 400. -	
Depreciation Charge on Equipment	DM 1. 000. -	
	Total:	DM 55. 300. -

November 30, 1961

O. Westphal

Dr. Otto Westphal
Professor of Biochemistry
and Immunochemistry